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(54) Title: GENERATION OF XENOGENEIC ANTIBODIES

(57) Abstract

The subject invention provides non-human mammalian hosts characterized by inactivated endogenous Ig loci and functional human Ig loci for response to an immunogen to produce human antibodies or analogs thereof. The hosts are produced by multiple genetic modifications of embryonic cells in conjunction with breeding. Different strategies are employed for recombination of the human loci randomly or at analogous host loci. Chimeric and transgenic mammals, particularly mice, are provided, having stably integrated large, xenogeneic DNA segments. The segments are introduced by fusion with yeast spheroplasts comprising yeast artificial chromosomes (YACs) which include the xenogeneic DNA segments and a selective marker such as HPRT, and embryonic stem cells.

GENERATION OF XENOGENEIC ANTIBODIES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of Application Serial No. 07/919,297 filed July 24, 1992 which was a continuation-in-part of Application Serial No. 07/610,515 filed November 8, 1990 which was a continuation-in-part of Application Serial No. 07/466,008 filed January 12, 1990, the entire disclosures of which are all incorporated herein by reference.

INTRODUCTION

Technical Field

The field of this invention is the production of xenogeneic specific binding proteins in a viable mammalian host.

Background

The ability to produce transgenic animals has been revolutionized with the advent of the ability to culture murine embryonic stem cells, and to introduce genetic modifications in these cells for subsequent transmission to the mouse germline. Thus one has the opportunity to modify endogenous genes to produce animal strains capable of producing novel products by introduction of foreign genes into the host, particularly human genes to produce xenogeneic binding proteins. The expression of such genes *in vivo* in an animal model may provide for investigation of the function of the gene, the regulation of gene expression, its processing, response to various agents and the like. In addition, animals with new phenotypes,

Because of the relationship between the sequence of the constant region and the species from which the antibody is produced, the introduction of a xenogeneic antibody into the vascular system of the host can produce an immune response. Where the xenogeneic antibody is introduced repetitively, in the case of chronic diseases, it becomes impractical to administer the antibody, since it will be rapidly destroyed and may have an adverse effect. There have been, therefore, many efforts to provide a source of syngeneic or allogeneic antibodies. One technique has involved the use of recombinant DNA technology where the genes for the heavy and light chains from a host were identified and the regions encoding the constant region isolated. These regions were then joined to the variable region encoding portion of other immunoglobulin genes from another species directed to a specific epitope.

While the resulting chimeric partly xenogeneic antibody is substantially more useful than using a fully xenogeneic antibody, it still has a number of disadvantages. The identification, isolation and joining of the variable and constant regions requires substantial work. In addition, the joining of a constant region from one species to a variable region from another species may change the specificity and affinity of the variable regions, so as to lose the desired properties of the variable region. Also, there are framework and hypervariable sequences specific for a species in the variable region. These framework and hypervariable sequences may result in undesirable antigenic responses.

It would therefore be more desirable to produce allogeneic antibodies for administration to a host by immunizing the host with an immunogen of interest. For primates, particularly humans, this approach is not practical. The human antibodies which have been produced have been based on the adventitious presence of an available spleen, from a host which had been previously

chromosomes containing human DNA fragments. Yeast artificial chromosome vectors are described by Burke et al., Science 236:806-812 (1987). Pavan et al., Mol. and Cell. Biol. 10(8):4163-4169 (1990) describe the introduction of a neomycin resistance cassette into the human-derived insert of a yeast artificial chromosomes using homologous recombination and transfer into an embryonal carcinoma cell line using polyethylene glycol-mediated spheroplast fusion. Pachnis et al., Proc. Nat. Acad. Sci. USA 87:5109-5113 (1990), and Gnirke et al., EMBO Journal 10(7):1629-1634 (1991), describe the transfer of a yeast artificial chromosome carrying human DNA into mammalian cells. Eliceiri et al., Proc. Nat. Acad.USA 88:2179-2183 (1991), describe the expression in mouse cells of yeast artificial chromosomes containing human genes. Huxley et al., Genomics 9:742-750 (1991) describe the expression in mouse cells of yeast artificial chromosomes containing the human HPRT gene. Mortensen et al., Mol. and Cell. Biol. 12(5):2391-2395 (1992) describe the use of high concentrations of G418 to grow heterozygous embryonic stem cells for selection of homozygous mutationally altered cells. Yeast protoplast fusion with mouse fibroblasts is described by Traver et al., Proc. Nat. Acad. Sci. USA 86:5898-5902 (1989) and Pachnis et al., Proc. Nat. Acad. Sci. USA 87:5109-5113 (1990). Davies et al., Nucl. Acids Res. 20:2693-2698 (1992) describe targeted alterations in YACs. Zachau, Biol. Chem. 371:1-6 (1990) describes the human immunoglobulin light (kappa) (IgK) locus; Matsuda et al., Nature Genetics 3:88-94 (1993) and Shin et al., EMBO 10:3641-3645 (1991) describe the cloning of the human immunoglobulin heavy (IgH) locus in YACs.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram of the inactivation vector for the mouse heavy chain J region, as described in Example I, infra.

Figure 2 is a diagram of the DNA restriction map for the plasmid pmHδJ and the targeted mouse heavy chain J genes, as described in Example II, infra.

Figure 3 is a flow cytometry plot of antibody staining for IgM allotypes in mouse strains, as described in Example II, infra.

Figure 4 is a flow cytometry histogram of antibody staining for IgM allotypes in mouse strains, as described in Example II, infra.

Figure 5 is a diagram of the inactivation vector for the mouse immunoglobulin kappa constant region genes, as described in Example III, infra.

Figure 6 is a diagram of the derivation of the plasmid pK.TK/Neo, as described in Example III, infra.

Figure 7 is a diagram of the restriction map of the light chain targeted locus, as described in Example III, infra.

Figure 8 is a diagram of the targeting vector for inactivation of the kappa light chain J and constant regions and design of the targeting experiment as described in Example IV, infra.

Figure 9 is a diagram of the construction of vectors for inactivating the kappa light chain J and constant regions as described in Example IV, infra.

Figure 14 A, B, C demonstrates the stable retention of yHPRT during in vitro ES cell differentiation and transmission through the mouse germline, as described in Example VI, infra (A: a, b = embryoid bodies; and differentiated cell types:c = blood islands; d=contracting muscle; e= neuronal cells; f = neural tubules formed by ESY clones; B: Southern blot analysis of DNA extracted from differentiated ESY 5-2, 3-6, 8-5 and 8-6 (20 μg) and yHPRT in AB1380 (40 ng) using a = human Alu probe; b = yeast Ty sequences; C: Southern blot analysis of tail DNA (20 μg) from 2 agouti offspring (4-2 and 4-3) derived from ESY chimeric male 394/95-2 using a = human Alu and b = Ty sequences; shorter exposures (12 hr) of 8-6 and yHPRT probed with Ty are shown (II).

Figure 15 A and B are a photograph of an electrophoresis gel showing the expression of the human HPRT gene in various mouse tissues, as described in Example VI, infra (15 A = detection of human HPRT mRNA using reverse transcription-PCR in ES, ESY 3-1 and Hut 78 cells, spleen and liver from control mice or ESY 4-3 agouti offspring; 15 B = detection of mouse γ-interferon receptor mRNA by RT-PCR in samples from 15 A; M = size marker).

Figure 16 is a diagram of the human immunoglobulin heavy chain locus, and a human heavy chain replacement YAC vector, as described in Example VII, infra.

Figure 17 is a diagram of a mouse breeding scheme, as described in Example VIII, infra.

Figure 18 depicts the genotypes of some of the host animals produced by the methods of the invention.

Inactivation of the endogenous host immunoglobulin loci is achieved by targeted disruption of the appropriate loci by homologous recombination in the host cells, particularly embryonic stem cells or pronuclei of fertilized mouse oocytes. The targeted disruption can involve introduction of a lesion or deletion in the target locus, or deletion within the target locus accompanied by insertion into the locus, for example, insertion of a selectable marker. In the case of embryonic stem cells, chimeric animals are generated which are derived in part from the modified embryonic stem cells and are capable of transmitting the genetic modifications through the germ line. The mating of hosts with introduced human immunoglobulin loci to strains with inactivated endogenous loci will yield animals whose antibody production is purely xenogeneic, e.g. human.

In a second, alternative strategy, at least portions of the human heavy and light chain immunoglobulin loci are used to directly replace the corresponding endogenous immunoglobulin loci by homologous recombination in embryonic stem cells. This results in simultaneous inactivation and replacement of the endogenous immunoglobulin. This is followed by the generation of chimeric animals in which the embryonic stem cell-derived cells can contribute to the germ line.

These strategies are based on the known organization of the immunoglobulin chain loci in a number of animals, since the organization, relative location of exons encoding individual domains, and location of splice sites and transcriptional elements is understood to varying degrees. In the human, the immunoglobulin heavy chain (IgH_{hu}) locus is located on chromosome 14. In the 5' - 3' direction of transcription, the locus comprises a large cluster of variable region genes (V_H), the diversity (D) region genes, followed by the joining (J_H) region genes and the constant (C_H) gene cluster. The size of the locus is estimated to be about from 1,500 to about 2,500

into a single chromosome in a host cell. This eliminates the need for breeding of animals containing individual human Ig genes in order to generate a host capable of producing fully human immunoglobulins. For example, a strain of yeast containing a single YAC is targeted with a vector such as pLUTO (described infra) to introduce a mammalian selectable marker such as HPRT, and a yeast selectable marker such as LYS2 into an arm of the YAC. Chromosomal DNA from the targeted strain is then used to transform a second, usually haploid, lys2 mutant yeast strain containing a second, different YAC. Lys+ colonies are then analyzed by pulsed-field gel electrophoresis (PFGE) to identify clones harboring the two YACs and to confirm that they are unaltered in size. Additional YACs with different selectable markers, for example ADE2 (if the host is an ade2 mutant), can subsequently be added by transformation. Alternatively, a YAC-containing strain of yeast is targeted with a vector such as pLUTO to introduce a mammalian selectable marker (e.g. HPRT), as above, and then mated to a second YAC-containing strain of opposite mating type. The presence of the two YACs is then confirmed in the diploid yeast cells as described above. The diploid yeast strain is used directly for fusion or put through meiosis and ascosporogenesis (sporulation) using standard procedures. The meiotic products are then screened to identify a haploid clone containing the two YACs. With either approach described above, the second YAC can be targeted with HPRT or another selectable marker prior to introduction of the first YAC. Also, if each YAC contains a different yeast selectable marker, maintenance of both YACS during strain propagation may be genetically selected. Fusion with ES cells is then carried out in the same manner as with yeast cells containing a single YAC. Because many yeast chromosomes may integrate along with the YAC, it is expected that a substantial portion of ES clones expressing the mammalian selectable marker present in one YAC (e.g. HAT^R clones

Because there are two heavy chain alleles and two light chain loci, kappa and lambda, each with two alleles, although one may choose to ignore the lambda loci, there will have to be multiple transformations which result in inactivation of each of the alleles. Homologous recombination may be employed to functionally inactivate each of the loci, by introduction of the homologous DNA via a construct that can disrupt or delete the target locus into embryonic stem cells, followed by introduction of the modified cells into recipient blastocysts. Subsequent breeding allows for germ- line transmission of the inactivated locus. One can therefore choose to breed heterozygous offspring and select for homozygous offspring from the heterozygous parents.

In the second, alternative strategy described above, the number of steps may be reduced by providing at least a fragment of the human immunoglobulin locus within the construct used for homologous recombination with the analogous endogenous immunoglobulin, so that the human locus is substituted for at least a part of the host immunoglobulin locus, with resulting inactivation of the host immunoglobulin subunit locus. Of particular interest is the use of transformation for a single inactivation, followed by breeding of the heterozygous offspring to produce a homozygous offspring. Where the human locus is employed for substitution or insertion into the host locus for inactivation, the number of transformations may be limited to three transformations and as already indicated, one may choose to ignore the less used locus and limit the transformations to two transformations. Alternatively, one may choose to provide for inactivation as a separate step for each locus, employing embryonic stem cells from offspring which have previously had one or more loci inactivated. In the event that only transformation is used and the human locus is integrated into the host genome in random fashion, a total of eight or more transformations may be required.

In the targeting construct, upstream and/or downstream from the target gene, may be a gene which provides for identification of whether a homologous double crossover has occurred (negative selection). For this purpose, the Herpes simplex virus thymidine kinase gene may be employed, since cells expressing the thymidine kinase gene may be killed by the use of nucleoside analogs such as acyclovir or gancyclovir, by their cytotoxic effects on cells that contain a functional HSV-tk (Mansour et al., Nature 336:348-352 (1988)). The absence of sensitivity to these nucleoside analogs indicates the absence of the HSV-thymidine kinase gene and, therefore, where homologous recombination has occurred, that a double crossover has also occurred.

While the presence of the marker gene in the genome will indicate that integration has occurred, it will still be necessary to determine whether homologous integration has occurred. This can be achieved in a number of ways. For the most part, DNA analysis by Southern blot hybridization will be employed to establish the location of the integration. By employing probes for the insert and the sequences at the 5′ and 3′ regions flanking the region where homologous integration would occur, one can demonstrate that homologous targeting has occurred.

PCR may also be used with advantage in detecting the presence of homologous recombination. PCR primers may be used which are complementary to a sequence within the targeting construct and complementary to a sequence outside the construct and at the target locus. In this way, one can only obtain DNA molecules having both the primers present in the complementary strands if homologous recombination has occurred. By demonstrating the expected size fragments, e.g. using Southern blot analysis, the occurrence of homologous recombination is supported.

The targeting construct may further include a replication system which is functional in the host cell. For the most part, these replication systems will involve

target locus, one can identify cells in which homologous recombination has occurred to inactivate a copy of the target locus.

The above described process may be performed first to inactivate a heavy chain locus in an embryonic stem cell whereby the cells are microinjected into host blastocysts which develop into a chimeric animal. The chimeric animals are bred to obtain heterozygous hosts. Then, by breeding of the heterozygous hosts, a homozygous host may be obtained or embryonic stem cells may be isolated and transformed to inactivate the second IgH locus, and the process repeated until all the desired loci have been inactivated. Alternatively, the light chain locus may be the first to be inactivated. For complete elimination of the ability to produce light chain immunoglobulin, it is desirable to inactivate both the lambda and the kappa light chain immunoglobulin loci. At any stage, the xenogeneic loci may be introduced.

As already indicated, the target locus may be substituted with the analogous xenogeneic locus. In this way, the xenogeneic locus will be placed substantially in the same region as the analogous host locus, so that any regulation associated with the position of the locus will be substantially the same for the xenogeneic immunoglobulin locus. For example, by isolating the variable region of the human IgH locus (including V, D, and J sequences), or portion thereof, and flanking the human locus with sequences from the murine locus, preferably sequences separated by at least about 5 kbp, in the host locus, preferably at least about 10 kbp in the host locus, one may insert the human fragment into this region in a recombinational event(s), substituting the human immunoglobulin locus for the endogenous variable region of the host immunoglobulin locus. In this manner, one may disrupt the ability of the host to produce an endogenous immunoglobulin subunit, while allowing for the promoter of the human immunoglobulin locus to be activated

the host then contains the human IgH and the human Ig kappa and/or lambda loci would allow for the production of purely human antibody molecules without the production of host or host/human chimeric antibodies. Such a host strain, by immunization with specific antigens, would respond by the production of murine B-cells producing specific human antibodies, which B-cells could be fused with murine myeloma cells or be immortalized in any other manner for the continuous stable production of human monoclonal antibodies. Methods are well known in the art for obtaining continuous stable production of monoclonal antibodies.

The subject methodology and strategies need not be limited to producing complete immunoglobulins, but provides the opportunity to provide for regions joined to a portion of the constant region, e.g., C_{H1}, C_{H2}, C_{H3}, or C_{H4}, or combination thereof. Alternatively, one or more of the exons of the C_H and C_k or C_λ regions may be replaced or joined to a sequence encoding a different protein, such as an enzyme, e.g., plasminogen activator, superoxide dismutase, etc.; toxin, e.g., ricin, abrin, diphtheria toxin, etc.; growth factor; cytotoxic agent, e.g., TNF; receptor ligand, or the like. See, for example, WO 89/07142; WO 89/09344; and WO 88/03559. By inserting the protein of interest into a constant region exon and providing for splicing of the variable region to the modified constant region exon, the resulting binding protein may have a different C-terminal region from the immunoglobulin. By providing for a stop sequence with the inserted gene, the protein product will have the inserted protein as the C-terminal region. If desired, the constant region may be entirely substituted by the other protein, by providing for a construct with the appropriate splice sites for joining the variable region to the other protein.

The B-cells from the transgenic host producing immunoglobulin or immunoglobulin analog may be used for

VI. Animals heterozygous for inactive endogenous heavy and light chain immunoglobulin genes and hemizygous for foreign, preferably human, heavy chain immunoglobulin genes obtained by crossbreeding animals of category III with animals from category V (animals homozygous for the inactive endogenous loci and homo- or hemizygous for the foreign gene are obtained by interbreeding);

VII. Animals heterozygous for inactive endogenous heavy and light chain immunoglobulin genes and hemizygous for foreign, preferably human, light chain immunoglobulin genes obtained by crossbreeding animals of category IV with animals from category V (animals homozygous for the inactive endogenous loci and homo- or hemizygous for the foreign gene are obtained by interbreeding);

VIII. Animals homozygous or heterozygous for inactive endogenous heavy and light chain immunoglobulin genes and hemizygous for foreign, preferably human, light and heavy chain immunoglobulin genes, obtained by crossbreeding animals of category VI and VII (animals homozygous for the inactive endogenous loci and homo- or hemizygous for the foreign gene are obtained by interbreeding);

In a preferred embodiment, the homozygous animals of category VIII are used to produce human antibodies.

IX. Animals homozygous for functional endogenous heavy and light chain immunoglobulin genes and hemizygous for foreign, preferably human, heavy and light chain immunoglobulin genes, obtained by crossbreeding animals of category III and IV (homozygous animals are obtained by interbreeding);

X. Animals heterozygous for an inactive endogenous heavy chain immunoglobulin gene and hemizygous for foreign, preferably human, heavy and light chain immunoglobulin genes, obtained by crossbreeding animals of category II and IX (animals homozygous for the inactive endogenous loci and homo- or hemizygous for the foreign gene are obtained by interbreeding).

the production of antibodies, provide for specific combinations of transcription factors, provide for metabolic systems, introduce dominant mutations or complement recessive mutations. The xenogeneic DNA may be modified when present in a YAC. Because homologous recombination is efficient in yeast, giving a high ratio of site-specific integration of homologous DNA, where the homologous DNA flanks other DNA of interest, one is able to modify the xenogeneic DNA before introduction into an ES cell. In this way, one can introduce defective genes into the host which express defective proteins to mimic diseased states of the xenogeneic host, to study various mechanisms of the interaction of defective proteins with other xenogeneic proteins or endogenous proteins, or to study genes or gene systems.

In general, to transfer large DNA segments, as described in detail herein, YACs are employed which comprise a yeast centromere, an origin of replication and telomeres bounding the DNA of interest. Various centromeres or telomeres may be used, particularly the centromeres from yeast chromosomes 4 and 5. The YAC has a marker which allows for selection or screening of cells into which the YAC becomes integrated. Not all markers allow for efficient selection. Particularly, the HPRT gene, more particularly human HPRT, is found to permit efficient selection of HPRT-deficient ES cells carrying the YAC. Other known selectable or screenable markers include hygromycin, neomycin, β-gal, and GPT. The ES cell may be derived from any non-human host, from which ES cells are available, and can be expanded in culture, which remain viable and functional, for which a marker for selection exists, and where the ES cell can be introduced into an embryo and can repopulate the host, including the germline. For the most part this capability has been established with rodents, e.g. mice and rats, and to a lesser extent with guinea pigs. Mice have been used for the production of antibodies or B-lymphocytes for

Those yeast hosts carrying the YAC may then be used as a source of the YAC for introduction into the ES cell. Transfer of the YAC is efficiently achieved by preparing yeast spheroplasts in accordance with conventional ways. By degrading the outer wall, under mild conditions, in an isotonic medium, spheroplasts are produced in high yield. Exponentially growing ES cells are protease-treated, e.g. trypsinized, and combined with the spheroplasts. Conveniently, a pellet of yeast spheroplasts can be prepared and the ES cells are spun with the pellet and exposed to a fusogenic agent such as PEG for 1-2 minutes. The cells are then resuspended and incubated in appropriate serrum-free medium. The cells are then plated onto feeder cells, followed by selection in accordance with the selective marker. For the HPRT gene, HAT medium may be employed for selection. Surviving fusion colonies are then, picked, expanded and analyzed. Analysis may be performed by restriction enzyme analysis, combined with Southern blotting or pulsed-field gel electrophoresis, or by the polymerase chain reaction (PCR), employing appropriate primers, at least one of which is complementary to the DNA insert, and probing with repetitive sequences present in the xenogeneic DNA, such as Alu, for detection of human DNA sequences. Ty, Y', rDNA, delta sequences are used to probe for for yeast sequences. Probes for YAC ends are used to confirm integrity of the YAC. Those cells that demonstrate the intact or substantially intact YAC DNA integrated into the host genome are then used in the next steps. In some clones, only a portion or little or none of the yeast DNA becomes integrated into the mouse genome. The integrated yeast DNA ranges from more than about 90% of the original yeast genome to less than about 10% .

In a preferred embodiment, efficient production of transgenic non-human hosts is provided using a process which integrates large, at least 100 kb, xenogeneic DNA fragments, in substantially intact form, into a host

injection, at least 1 and no more than about 10 of the blastocysts are returned to each uterine horn of pseudo-pregnant females. The females proceed to term and the resulting chimeric animals can be analyzed for the presence of the YAC in their somatic cells. By "chimeric" is meant an animal that carries cells derived from more than one source, e.g. from the host and another animal. For example, in the present invention a chimeric murine animal contains a genetically engineered modification, particularly a human gene, in some of its cells, e.g. in cells that develop from the modified embryonic stem cells. The presence of the integrated YAC in chimeric hosts that are generated is then analyzed. The chimeric hosts are evaluated for germline transmission of the ES cell genome by mating, for example chimeric mice are mated with C57BL/6J mice. Chimeric hosts may be bred with non-chimeric hosts, either syngeneic or allogeneic, to screen for chimeras that carry the YAC in their germ cells. Offspring that are heterozygous for the genetic modification are then interbred to produce progeny that are homozygous for the modification, stably transmitting the functioning YAC construct to their progeny.

The method of the invention for introduction of large xenogeneic DNA segments into a non-human host, particularly a rodent and usually a murine animal, provides for stable integration of the DNA. Genes in the inserted DNA are found to be functional and the resulting chimeric hosts are able to provide for germline transmission of the integrated DNA. After breeding of the chimeric host, transgenic heterozygous hosts are produced and are mated to produce a homozygous animal that may be used for a wide variety of purposes, including production of products, such as binding proteins, for example immunoglobulins, for screening of various drugs, for gene therapy, for example to complement for recessive genetic disorders, to study various diseases, to study

as described (Doetschman et al. (1985), J. Embryol. Exp. Morphol. 87:27-45). The embryonic fibroblasts are prepared from embryos from C57BL/6 females that are mated 14 to 17 days earlier with a male homozygous for a neomycin transgene (Gossler et al. (1986), PNAS 83:9065-9069). These cells are capable of growth in media containing G418. Electroporation conditions are described by (Boggs et al. (1986), Ex. Hematol. (NY) 149:988-994). ES cells are trypsinized, resuspended in culture media at a concentration of $4x10^7$/ml and electroporated in the presence of the targeting DNA construct at a concentration of 12nM in the first experiment and 5nM DNA in the second. A voltage of 300 V with a capacitance of 150-250 μF is found optimal with an electroporation cell of 5 mm length and 100 mm^2 cross-section. $5x10^6$ electroporated cells are plated onto mitomycin-treated fibroblasts in 100 mm dishes in the presence of Dulbecco's modified Eagle's media (DMEM) supplemented with 15% fetal bovine serum (FBS) and 0.1 mM 2-mercaptoethanol. The media is replaced 24 hrs after electroporation with media containing 200 μg/ml G418.

ES colonies resulting 10-14 days after electroporation are picked with drawn out capillary pipettes for analysis using PCR. Half of each picked colony is saved in 24-well plates already seeded with mitomycin-treated feeder cells. The other halves, combined in pools of 3-4, are transferred to Eppendorf tubes containing approximately 0.5 ml of PBS and analyzed for homologous recombination by PCR. Conditions for PCR reactions are essentially as described (Kim and Smithies (1988), Nucleic Acids Res. 16:8887-8893). After pelleting, the ES cells are resuspended in 5 μl of PBS and are lysed by the addition of 55 μl of H_2O to each tube. DNAses are inactivated by heating each tube at 95°C for 10 min. After treatment with proteinase K at 55°C for 30 min, 30 μl of each lysate is transferred to a tube containing 20 μl of a reaction mixture including PCR

about 2.8 kb and about 1.1 kb fragments, located 5′ and 3′ to the neomycin gene, respectively.

B. Culturing, Electroporation, and Selection of ES cells

The ES cell line E14TG2a (Koller and Smithies (1989), PNAS USA, 86:8932-8935) was cultured on mitomycin C-treated embryonic fibroblast feeder layers as described (Koller and Smithies (1989), PNAS USA, 86:8932-8935). ES cells were trypsinized, resuspended in HBS buffer (pH 7.05; 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.7 mM Na_2HPO_4, 21 mM HEPES pH 7.1) at a concentration of $2x10^7$/ml and electroporated in the presence of 50 μg/ml of the linearized inactivation vector. Electroporation was carried out with a BioRad Gene Pulser using 240 volts and 500 μF capacitance. $5x10^6$ electroporated cells were plated onto mitomycin C-treated fibroblasts in 100 mm dishes in the presence of Dulbecco's modified Eagle's media (DMEM) supplemented with 15% fetal bovine serum and 0.1 mM 2-mercaptoethanol. The media was replaced 24 hr after electroporation with media containing 200 μg/ml G418. G418-resistant ES colonies resulting from growth 12-14 days after electroporation were picked with drawn out capillary pipettes for analysis using the polymerase chain reaction (PCR). Half of each picked colony was transferred to an individual well of a 24-well plate, already seeded with mitomycin C-treated feeder cells. The other halves, combined in pools of four, were transferred to Eppendorf tubes containing 0.3 ml of PBS and cell lysates were prepared for PCR analysis as described by Joyner et al (1989) Nature, 338:153-155. The PCR reaction included 5-20 μl of the cell lysate, 1 μM of each primer, 1.5 U of Taq polymerase and 200 μM of dNTPs. The PCR amplification employed 45 cycles using a thermal cycler (Perkin-Elmer Cetus), with 1 min. melt at 94°C, 2 min. annealing at 55°C, and 3 min. extension at 72°C. The two priming oligonucleotides are ACGGTATCGCCGCTCCCGAT and AGTCACTGTAAAGACTTCGGGTA, which

probe, which is about 1.9 kb longer than the equivalent fragment in the native locus, due to the loss of two HindIII sites located in the deleted J gene region (see Figure 2C). Southern analysis of each of the 5 positive clones by HindIII digestion gave a pattern which indicated that one of the two copies of the heavy chain J genes had been disrupted. Three labelled fragments were detected: one fragment (about 760 bp), identical in size to that present in untreated cells at the same intensity, one fragment (about 2.3 kb) identical in size to that present in untreated cells, but of decreased intensity in the PCR positive clone, and an additional fragment about 4.2 kb, the size predicted for an homologous recombination event, present only in the PCR-positive clones. Similarly, the replacement of the J genes by the neomycin gene by an homologous recombination event results in a loss of one SacI site and the appearance of a fragment, detectable with the EcoRI-PstI probe, which is about 570 bp smaller than the equivalent fragment in the native locus (see Figure 2C). Southern analysis of the clones by SacI digestion gave the expected pattern of one native and one targeted allele: about 4.0 kb fragment, identical in size to that detected in untreated cells, but of decreased intensity in the 5 positive clones, and an additional fragment of about 3.4 kb, the size predicted for a targeted homologous recombination event, present only in the identified clones. Rehybridization of the Southern blots with a probe for the neomycin gene showed that only the 4.2 kb and 3.4 kb fragments, resulting from the HindIII and the SacI digestion, respectively, hybridized to the probe as predicted by the targeting event.

D. Generation of chimeric mice with J_H deletions

Three and a half day old C57BL/6J (Jackson Laboratories, Bar Harbor, ME) blastocysts were obtained from 4-5 week old superovulated females as described by Koller, et al. 1989 (supra). ES cells were trypsinized,

matings were analyzed for the presence of the two targeted heavy chain alleles by Southern blot analysis.

E. Analysis of B cells from chimeric mice

If deletion of the J_H region is sufficient to inactivate the heavy chain locus, then it should result in complete block of development of IgM-expressing B cells and of antibody production. Mice which are heterozygous at the J_H locus carry one intact and functional heavy chain allele, derived from the C57BL/6J parent, and one J_H-deleted heavy chain allele which is derived from the ES cells (129/Ola strain). The 129 and B6 strains differ in Ig heavy chain allotypes. The ES-derived B cells (IgM^a allotype) can be distinguished from B6-derived B cells (IgM^b allotype) with allotype-specific monoclonal antibodies, using flow cytometry analysis of antibody expressing B.

The specificity of these antibodies is shown in Figure 3 (A-C). Peripheral blood lymphocytes were stained with antibodies to the B cell specific marker, B220, and with antibodies to the IgM allotype. B cells from C57BL/6J mice stained with antibodies directed against the IgM^b allotype but not the IgM^a allotype (Figure 3B). B cells derived from 129/Ola mice stained with antibody against the IgM^a allotype, but not the IgM^b allotype (Figure 3A). In heterozygous (a/b F1) mice carrying one intact ES-derived heavy chain allele and one intact C57BL/6J-derived heavy chain allele, both allotypes were present in equal amounts (Figure 3C).

When B cells from mice which were heterozygous for the J_H deletion were analyzed, where the J_H deleted heavy chain allele was from the 129/Ola parent, there were no cells positive for the IgM^a allotype. All B cells were IgM^b positive, from the intact C57BL/6J heavy chain allele (Figure 3D). These results indicated that the J_H-deleted heavy chain locus is inactivated and cannot encode a functional IgM antibody.

G. Analysis of B cells from homozygous chimeras

B cells from chimeric mice were analyzed to determine the effect of J_H deletion on B cell development and antibody production. Lymphocytes from the ES cell line (129/Ola) can be distinguished from blastocyst-derived (C57BL/6J) lymphocytes by a monoclonal antibody to the Ly-9.1 marker, which is found on lymphocytes of 129 origin, but not those of B6 origin. In addition, the two strains differ in their IgM allotype, as previously described.

The chimeras analyzed had been derived from wild-type E14TG2a ES cells (WT), or from ES cells that were heterozygous (ES110-1, ES65-1) or homozygous (ESDK207) at the targeted J_H region. Peripheral blood mononuclear cells were stained with antibodies to the B cell specific marker B220, and with antibodies to either Ly-9.1 or IgM allotypes, and then analyzed by two-color flow cytometry. To evaluate chimerism in the T cell lineage, the cells were stained with antibody for the T cell marker Thy 1.2, and with anti-Ly-9.1 antibody. Staining of cells from the parental mouse strains provided controls for the specificity and sensitivity of the assay.

Mice with similar degrees of chimerism, as judged by coat color, were compared. ES-derived B and T cells were detected in the peripheral blood of chimeric mice generated from the wild-type E14TG2a ES cells, confirming the ability of this cell line to give rise to lymphoid cells in vivo. Analysis of chimeras generated from single J_H-targeted ES65-1 and ES110-1 cells demonstrated the presence of $B220^+/IgM^{a-}/Ly\text{-}9.1^+$ B cells containing a single, intact, ES cell-derived Ig heavy chain locus.

In contrast to the WT and single deletion chimeras, mice generated from the homozygous mutant ESDK207 cell line lacked $Ly\text{-}9.1^-/B220^+$ or $IgM^{a+}/B220+1$ B cells in peripheral blood. The observed lack of ESDK207-derived B cells was not due to a lack in lymphopoiesis, since ES-derived $Ly\text{-}9.1^+/B220^-$ cells represented 12% of the total

EXAMPLE III

Deletion of the Mouse Ig kappa light chain constant (C_κ) region

A. Construction of the replacement targeting vector

The kappa region was inactivated with a replacement type vector, which was designed to delete the constant region of the kappa locus, and replace it with the G418 drug resistance marker through homologous recombination. Homologous recombination was driven by regions of homology which flank the constant region (see Figure 5).

A genomic library from 129/Ola mouse fetal liver DNA (Stratagene) cloned into lambda phage was screened for the presence of the mouse C_κ gene with a 1.6 kb HpaI/BamHI fragment (Steinmetz and Zachau (1980) Nucleic Acids Research 8:1693-1706) that spans the mouse kappa constant region. A lambda phage clone which hybridized to this probe was identified, then purified and used as a source of C_κ DNA. Analysis of the phage DNA showed that the kappa constant region probe hybridized to a 5.6 kb SphI/BamHI fragment. This fragment contained the kappa J region genes, an intronic enhancer element and the kappa constant region. It was then isolated and subcloned into the SphI and BamHI sites of the plasmid pUC218 to give the plasmid pUC218/5.6kappa.

In order to construct the deletion vector, fragments containing the 5′ region of the kappa constant region, a thymidine kinase gene for negative selection, a neomycin resistance gene and a 3′ region of homology to the kappa constant region were ligated together (see Figure 6).

A 4.0 kb SphI/Bsu361 fragment from the plasmid pUC218/5.6kappa was subcloned into the SphI and Bsu361 sites of the vector pSK.A to give the plasmid pSK.A/5′K. The vector pSK.A is a modification of pBluescript SK- which has a synthetic polylinker:

5′ GCATATGCCTGAGGTAAGCATGCGGTACCGAATTCTATAAGCTTGCGGCCGCAGCT
CATGCGTATACGGACTCCATTCGTACGCCATGGCTTAAGATATTCGAACGCCGGCG 3′

B. Electroporation of kappa deletion vector into ES cells

Purified plasmid DNA from pK.TK/Neo was cut with PvuI, extracted with phenol/chloroform and ethanol precipitated. The DNA was resuspended after precipitation at a concentration of 1 mg/ml in 10 mM Tris-HCl, 1 mM EDTA.

The embryonic stem cell line E14-1, a subclone of E14 (Hooper, et al. (1987) Nature 326:292-295) was cultured in DMEM 4.5 g/l glucose (J.R.H. Biosciences) supplemented with 15% heat inactivated fetal calf serum, recombinant murine leukemia inhibitory factor (ESGRO from Gibco BRL, 1000 U/ml), 0.1 mM β-mercaptoethanol, 2 mM glutamine and 100 U/ml penicillin at 37° C in 5% CO_2.

The cells were cultured on mitomycin-treated primary embryonic fibroblast feeder layers essentially as described (Koller and Smithies (1989) supra). The embryonic fibroblasts were prepared from day 14 embryos carrying the homozygous targeted mutation of β2-microglobulin (Koller and Smithies (1990) Science 248:1227-1230). These feeder cells are capable of growth in media containing G418.

At 80% confluency, the ES cells were prepared for electroporation by trypsinization, concentration by brief centrifugation and resuspension in HEPES-buffered saline at 2 x 10^7 cells/ml. The cells are equilibrated at room temperature, and linearized targeting vector DNA (20 μg) added. The mixture was electroporated at 960 μF and 250 V with a BioRad Gene Pulser. The cells were left to stand at room temperature for 10 minutes before plating onto 4 x 10 cm dishes of mitomycin-treated fibroblast feeders (3 x 10^6 feeder cells/plate). After incubation at 37° C for 48 hours, the cells were fed media containing 150 μg/ml G418 to select for neomycin resistance. After a further 48 hours the cells were fed media containing 150 μg/ml G418 and 2 μM gancyclovir (Syntex) to select for loss of the thymidine kinase gene.

genomic DNA, an about 15 kb fragment is detected in the native allele, and an about 5 kb fragment from the targeted locus. The additional EcoRI site is introduced by the neo gene during homologous recombination targeting (see Figure 7).

D. Generation of germline chimeras

The unmodified E14-1 cells have been found to contribute to the germline at a high frequency after injection into C57BL/6J blastocysts. To generate germline chimeras containing the targeted kappa region, the targeted cell lines 1L2-850 and 1L2-972 were grown on primary feeder cells, then trypsinized and resuspended in injection medium, which consists of DMEM supplemented with 15% fetal calf serum, 20 mM HEPES (pH 7.3), antibiotics and β-mercaptoethanol. The ES cells were injected into each blastocyst, and the injected blastocysts then transferred to one uterine horn of a pseudopregnant female mouse. Chimeric pups were identified by chimeric coat color. Chimeric males were bred to C57BL/6J females, and germline transmission of the 129/Ola derived ES cells was detected by agouti coat color of the offspring.

One chimeric male from cell line 1L2-972 (about 40% ES cell derived as judged by its coat color), upon mating with C57Bl/6J females yielded germline transmission at a frequency of 25% as determined by the percent of agouti offspring. Chimeric males, about 40%, 70% and 90% chimeric, from cell line 1L2-850 yielded germline transmission at a frequencies of 90%, 63% and 33%, respectively. Among the agouti offspring generated from the 70% chimeric male from 1L2-850, eight F1 animals out of 12 tested were found to be heterozygous at the kappa locus for the targeted C_K mutation by Southern analysis (a Bgl II digest using the 1.2 kb Bam HI/Bgl II fragment described above as a probe) using genomic DNA derived from tail samples. Further breeding of a male and female from

Inactivation of the mouse immunoglobulin kappa light chain J and constant region

A. Design of the targeting experiment

The targeting vector was designed as a replacement type vector initially to delete the constant region as well as the J region of the kappa locus and replace it with three elements through homologous recombination using regions of homology flanking the constant region (Figure 8). A diphtheria toxin gene (A chain) flanking either or both regions of homology was included in some cases as a negative selectable marker. The three elements consisted of the G418 resistance drug marker, an additional DNA homology (ADH) sequence of mouse DNA homologous to a region of the kappa locus located upstream of the J region, and a thymidine kinase gene. As a result of the inclusion of the ADH sequence in the vector, this initial targeting placed a second copy of the ADH in the locus. This duplication was then used to effect a defined deletion of the sequences between the segments by applying selective pressure. In this case the cell deletes the thymidine kinase gene that lies between the two segments in order to survive gancyclovir selection.

B. Construction of the targeting vector

The regions of homology were derived from a 129 mouse fetal liver genomic library (Stratagene) which was screened using two probes, as described above in Example III. This subclone contained the J region, an intronic enhancer element and the constant region of the kappa light chain locus. The second probe was a 0.8 kb EcoRI fragment (Van Ness et al. (1981), Cell 27:593-602) that lies 2.8 kb upstream of the J region. Phage DNA from a lambda clone positive for this probe showed that the probe hybridized to a 5.5 kb SacI fragment which was subcloned into the SacI site of pBluescript SK⁻ (Stratagene) to give the plasmid pSK.5'kappa (Figure 8).

46:4660-4664) containing the diphtheria toxin A chain driven by the human metallothionein (hMTII) promoter was cloned into pBluescript SK- cut with XbaI and EcoRI to give the plasmid pSK.DT. The hMTII promoter of pSK.DT was replaced with the PGK promoter from pKJ1 (Tybulewicz et al. (1991), Cell 65:1153-1163). A 0.5 kb XbaI/PstI fragment from pKJ1 was joined to a 3.1 kb XbaI/NcoI fragment from pSK.DT using a PstI/NcoI adapter formed from the oligonucleotides 5'-GGGAAGCCGCCGC-3' and 5'-CATGGC GGCGGCTTCCCTGCA-3' to give the plasmid pSK.pgkDT. A 248 bp fragment containing the bovine growth hormone polyadenylation signal, obtained by PCR amplification of bovine genomic DNA using the oligonucleotide primers 5'-CAGGATCCAGCTGTGCCTTCTAGTTG-3' and 5'-CTGAGCTCTAGACCCATA GAGCCCACCGCA-3', was cloned into pCR1000 (Invitron Corp., San Diego, CA). The polyadenylation sequence was then cloned behind the DT gene as a HindIII/PvuII fragment into pSK.pgkDT cut with HindIII and HpaI to give the plasmid pSK.pgkDTbovGH. The DT gene cassette from pSK.pgkDTbovGH was moved as a 2.1 kb EcoRI/HindIII fragment into pSK.A cut with EcoRI and NotI using a HindIII/NotI adapter formed from the oligonucleotides 5'-AGCTGGAACCCCTTGC-3' and 5'-GGCCGCAAGGGGTTCC-3' to give the plasmid pSK.A/DT. Between the SphI and Bsu36I sites of both pSK.A and pSK.A/DT the 5' region of homology for the kappa locus was cloned. For this purpose a 4.0 kb SphI/Bsu361 fragment resulting from a partial Bsu36I digest followed by a complete SphI digest of plasmid subclone pUC218/5.6kappa was ligated to pSK.A or pSK.A/DT to give the plasmids pSK.A/5'K and pSK.A/DT/5'K, respectively. In the plasmid, pSK.A/DT/5'K, the 5'-end of the DT gene and kappa fragment were adjacent to each other running in the opposite transcriptional orientations.

The PGKtk gene from the plasmid pKJtk (Tybulewicz et al. (1991), Cell 65:1153-1163) was cloned as a 2.7 kb EcoRI/HindIII between the unique EcoRI and HindIII sites of pSK.B to give pSK.B/TK. A 0.8 kb EcoRI fragment used

DNA pellets were resuspended at a concentration of 1 mg/ml in 10 mM Tris-HCl, 1 mM EDTA(TE).

C. Introduction of DNA into cells

The embryonic stem cell line E14-1 was cultured as described above in Example III. The cells were equilibrated at room temperature, and DNA (20 μg) linearized with PvuI (as described above) was added. The mixture was electroporated as described above in Example III.

D. Analysis of constant region-targeted ES cells

After 7-10 days under drug selection with G418, the individual surviving colonies were each picked and dissociated in a drop of trypsin as described above in Example III.

Southern analysis was carried out using BgIII digested genomic DNA from each sample. A 2.3 kb fragment was detected from the native ES cell locus, while a larger 4.9 kb fragment was detected from a targeted ES cell locus (Figure 11), using as a probe the 1.2 kb BamHI/BgIII fragment isolated from the original phage DNA contiguous with the fragment used for the 3′ homology in the targeting vector. The fragment increased in size because the BgIII site in the BgIII/BamHI fragment was lost in the targeting plasmid due to the joining of a BgIII site to a BamHI site in the ligation, and a new BgIII site located in the thymidine kinase gene is introduced into the targeted locus.

From a screen by the Southern analysis described above, of a total of 103 clones derived from experiments using three different targeting plasmids, 5 cell lines were identified which carried the intended mutation (Table 1).

Table 1

C_K Light Chain Targeting Result in E14-1

kb EcoRI fragment used as the ADH in the targeting vectors, as 12.7 kb fragment was detected from the native ES cell locus, while a larger 15.8 kb fragment was detected from the constant region-targeted ES cell locus (Figure 11) using DNA from clone 653. The fragment increased in size because of the insertion of the tk gene, the ADH, and the neo gene into the 12.7 kb BamHI fragment. There was also a new BamHI site introduced at the 3' end of the neo gene. Using DNA from the J/constant region deleted cells, a 5.5 kb fragment was detected from the modified locus in addition to the 12.7 kb fragment from the untargeted allele as predicted from analysis of the restriction map. From this screen by Southern analysis of 2 clones produced from 1.5 x 10^6 ES cells plated (clone 653), one cell line (clone 653B) was identified which carried the intended deletion of the J and constant regions.

Further analysis of genomic DNA produced from clone 653B after being thawed and expanded re-confirmed the initial observations. Using the 0.8 kb EcoRI fragment, the deletion was checked with two other restriction digests which should cut outside of the excised region on the 5' and 3' ends of the targeting vector. Thus using this probe with a BgIII digest of the genomic DNA from the unexcised clone 653, a 2.6 kb fragment was detected from both the unmodified and modified alleles, whereas an additional 4.9 kb fragment was observed from the targeted allele only (Figure 11). This 4.9 kb fragment was the same as that detected with the 1.2 kb BamHI/BgIII fragment used previously. Using DNA from clone 653B, a BgIII digest revealed a 5.8 kb fragment in addition to the 2.6 kb fragment from the unmodified allele. A SacI digest of clone 653 DNA probed with the 0.8 kb EcoRI fragment showed a 5.5 kb fragment from both the unmodified and modified alleles and a 3.1 kb fragment from the targeted allele only (Figure 11). The 5.5 kb fragment was also detected in DNA from clone 653B and an additional

B. Introduction of YAC clones into embryos or ES Cells

High molecular weight DNA is prepared in agarose plugs from yeast cells containing the YAC of interest (i.e., a YAC containing the aforementioned SpeI fragment from the IgH locus). The DNA is size-fractionated on a CHEF gel apparatus and the YAC band is cut out of the low melting point agarose gel. The gel fragment is equilibrated with polyamines and then melted and treated with agarase to digest the agarose. The polyamine-coated DNA is then injected into the male pronucleus of fertilized mouse embryos which are then surgically introduced into the uterus of a psueudopregnant female as described above. The transgenic nature of the newborns is analyzed by a slot-blot of DNA isolated from tails and the production of human heavy chain is analyzed by obtaining a small amount of serum and testing it for the presence of Ig chains with rabbit anti-human antibodies.

As an alternative to microinjection, YAC DNA is transferred into murine ES cells by ES cell: yeast protoplast fusion (Traver et al., (1989) Proc. Natl. Acad. Sci., USA, 86:5898-5902; Pachnis et al., (1990), ibid 87: 5109-5113). First, the neomycin-resistance gene from pMC1Neo or HPRT or other mammalian selectable marker and a yeast selectable marker are inserted into nonessential YAC vector sequences in a plasmid. This construct is used to transform a yeast strain containing the IgH YAC, and pMC1Neo (or other selectable marker) is integrated into vector sequences of the IgH YAC by homologous recombination. The modified YAC is then transferred into an ES cell by protoplast fusion (Traver et al. (1989); Pachnis et al., 1990), and resulting G418-resistant ES cells (or exhibiting another selectable phenotype) which contain the intact human IgH sequences are used to generate chimeric mice. Alternatively, a purified YAC is transfected, for example by lipofection or calcium phosphate-mediated DNA transfer, into ES cells.

identified in the A287-C10 microtiter well. Hence, an earlier aliquot of the A287-C10 microtiter plate well was examined in order to search for the progenitor YAC under the assumption that it was lost during passaging of the library. The A287-C10 microtiter well was streaked out (Washington University, St. Louis, MO), and 2 of 10 clones analyzed contained a 230 kb IgH YAC with another apparently unrelated YAC. Clone 1 contained in addition the IgH YAC, an approximately 220 kb YAC and clone 3 in addition contained an approximately 400 kb YAC. The IgH YAC contained mu, the complete D profile (based on a BamHI digest, see below) and JH. The IgH YAC from clone 1 was physically separated from the unrelated YAC by meiotic segregation in a cross between A287-C10/AB1380 and YPH857 (genotype = MATα ade2 lys2 ura3 trp1 HIS5 CAN1 his3 leu2 cyh2, to yield A287-C10 (230 kb)/MP 313 (host genotype = MATα ade2 leu2 lys2 his3 ura3 trp1 can1 cyh2).

2. Targeting of the A287-C10 kb YAC with a mammalian selectable marker, HPRT:

A YAC right arm targeting vector called pLUTO (15.6 kb) was generated by subcloning a human HPRT minigene contained on a 6.1 kb BamHI fragment (Reid et al., Proc. Natl. Acad. Sci. USA 87:4299-4303 (1990)) into the BamHI site in the polylinker of pLUS (Hermanson et al., Nucleic Acids Research 19:4943-4938 (1991)). A culture of A287-C10/AB1380 containing both the 230 kb IgH YAC and an unrelated YAC was transformed with linearized pLUTO and Lys+ transformants were selected. The Lys+ clones were screened by colony hybridization for the presence of mu. One clone was identified which contained a single YAC of approximately 245 kb which hybridized to probes for mu, HPRT and LYS2.

Southern analysis of the 230 kb A287-C10 YAC targeted with pLUTO was carried out using a variety of probes to demonstrate the intact, unrearranged nature of the cloned, human IgH sequences. In most cases, the results of BamHI,

sizes (Ravetch et al., supra; Shin et al., supra): > 12 kb BamHI (approximately 17 kb expected); 0.9 kb EcoRI (0.9 kb expected) and approximately 12 kb HindIII (approximately 11 kb expected). WI38 gave the same-sized BamHI fragment as A287-C10. The JH and DHQ52 regions were sequenced from both of the deletion derivative YACs and both were in germline configuration. Delta was analyzed with an exon 1 PCR product (containing the approximately 160 bp region between primers D1B= 5′ CAA AGG ATA ACA GCC CTG 3′ and D1D = 5′ AGC TGG CTG CTT GTC ATG 3′); restriction fragments for A287-C10 were close to those expected from the literature (Shin et al., supra) and to those determined for WI38. The 3′ cloning site of the YAC may be the first EcoRI site 3′ of delta (Shin et al., supra) or another EcoRI site further 3′. VH gene probes for VH1, VH4 and VH6 (Berman et al., supra), and for VH2 (Takahashi et al., 1984, Proc. Nat. Acad. Sci. USA 81:5194-5198) were used to evaluate the variable gene content of the YAC. A287-C10 contains two VH1 genes that approximate the predicted sizes (Shin et al., supra; Matsuda et al., 1993, supra); restriction analysis with the three enzymes gave close to the expected fragment sies; e.g. with EcoRI observed bands are 3.4 and 7.8 kb (expected are 3.4 and 7.2 kb). The predicted size EcoRI fragments for VH4 (5.3 kb observed, 5.1 kb expected) and for VH6 (0.8 kb observed, 0.9 kb expected) (Shin et al., supra; Matsuda et al., supra) were present in A287-C10. The expected size EcoRI fragment was seen for VH2 (5.5 kb observed, 5.4 kb expected), but the BamHI and HindIII fragments were different from those predicted. Coincident hybridization of the BamHI and HindIII fragments with a pBR322 probe suggested that the EcoRI site which is at the 5′ end of the VH2 gene (Shin et al., supra) is the 5′ cloning site, thus eliminating the natural 5′ HindIII site and BamHI sites. The overall size of the YAC insert (estimated to be approximately 220 kb) fits well with the predicted size for an intact, unrearranged segment

(Lorenz et al., Molec. Immunol. 25:479-484 (1988)). PCR analysis of both IgK YACs as well as human genomic DNA for the following kappa locus sequences revealed the predicted band sizes: Kde (120 bp), CK (304 bp), C-J intronic enhancer (455 bp), JK1-5 (1204 bp), B3 VK (123 bp) and B1 VK pseudogene (214 bp). Sequences used to design PCR primers for the CK, JK and C-J enhancer regions are from Whitehurst et al., Nucl. Acids. Res. 20:4929-4930 (1992); Kde is from Klobeck and Zachau, Nucl. Acids. Res. 14:4591-4603 (1986); B3 is from Klobeck et al., Nucl. Acids. Res. 13:6515-6529 (1985); and B1 is from Lorenz et al., supra.

B. Introduction of 680 kb yHPRT YAC into ES Cells

1. Culture of yHPRT yeast strain and preparation of yeast spheroplasts

The 680 kb yHPRT is a YAC containing a functional copy of the human hypoxanthine phosphoribosyltransferase (HPRT) gene cloned from a YAC library, as described in Huxley, et al. (1991) Genomics 9:742-750. The yeast strain containing the yHPRT was grown in uracil and tryptophan deficient liquid media, as described in Huxley, et al. (1991) supra.

To prepare the yeast spheroplasts, a 400 ml culture of yeast containing yHPRT was spun down and the yeast pellet was washed once with water and once with 1 M sorbitol. The yeast pellet was resuspended in SPEM (1 M sorbitol, 10 mM sodium phosphate pH 7.5, 10 mM EDTA pH 8.0, 30 mM β-mercaptoethanol) at a concentration of 5 x 10^8 yeast cells/ml. Zymolase 20T was added at a concentration of 150 μg/ml of yeast cells, and the culture was incubated at 30°C until 90% of the cells were spheroplasts (usually for 15-20 minutes). The cells were washed twice in STC (1 M sorbitol, 10 mM Tris pH 7.5, 10 mM $CaCl_2$) and resuspended in STC at a concentration of 2.5 x 10^8/ml.

Natl. Acad. Sci. USA 80:477-481 (1983)) was used to confirm the presence of the human HPRT gene in ESY clones. The Alu probe was a 300 bp BamHI fragment from the BLUR8 Alu element in pBP63A (Pavan et al., Proc. Natl. Acad. Sci. USA 78:1300-1304 (1990)). The right and left vector arm probes were pBR322-derived BamHI-PvuII 1.7 and 2.7 kb fragments, respectively, which correspond to the vector sequences in pYAC4 (scheme a, b (Burke et al., in: Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology, Guthrie and Fink, eds., Academic Press, 194:251-270 (1991)). The 4.5 kb fragment, detected by the right arm probe, spans the region between the HindIII site at the telomere 5′ end and the first HindIII site within the human insert (scheme a). The 3 kb and 4.1 kb fragments detected by the left end probe correspond to the region between the HindIII site at the telomere end and the HindIII site 5′ of the yeast sequences, and the region spanning from the HindIII site 3′ of the centromere into the human insert, respectively (scheme b). The difference in the hybridization intensity of these two bands relates to the difference in the amount of homology between these fragments and the probe. The yeast Ty repetitive probe (Philippsen et al., in Gene Expression in Yeast, Proceedings of the Alko Yeast Symposium, Helsinki, Korhola and Vaisanen, eds., Foundation for Biotechnical and Industrial Fermentation Research, 1:189-200 (1983)) was a 5.6 kb XhoI fragment isolated from Ty1-containing pJEF742 which could also detect the 3′ HindIII fragment of Ty2, due to the homology between the two elements. The LYS2 gene probe was a 1.7 BamHI fragment from pLUS (Hermanson et al., Nuc. Acids. Res. 19:4943-4948 (1991)).

Hybridization with a human HPRT probe (full length 1.6 kb cDNA probe) demonstrated that all the clones analyzed contained the same 15, 7 and 5 kb exon-containing fragments of the human HPRT gene as the yHPRT YAC. Reprobing the same blots with a human repetitive Alu

human sequences. Photomicrographs of representative metaphase spreads (Figure 13 A, B, C) or interphase nuclei (Figure 13D) from ESY 8-7 cells (Figure 13 A, B) hybridized with biotinylated human genomic sequences and ESY 8-6 cells (Figure 13 C, D) hybridized with biotinylated yeast repeated DNA sequences. The human probe was generated from human genomic placental DNA (Clontech, Palo Alto, CA). The yeast probe consisted of a mix of DNA fragments encoding the yeast repeated elements; delta (a 1.08 kb Sau3A fragment of pdelta6 (Gafner et al., EMBO J. 2:583-591 (1983)) and Ty (a 1.35 kb EcoRI-SaII fragment of p29 (Hermanson et al., Nuc. Acids. Res. 19:4943-4948 (1991)), the rDNAs (a 4.6 kb BgIIIk-A L90 and a 4.4 kb BgIII-B L92 fragment (Keil and Roeder, Cell 39:377-386 (1984)), and the Y' telomere elements (2.0 and 1.5 kb BgIII-HindIII fragments of p198 (Chan and Tye, Cell 33:563-573 (1983)). Hybridization of sequences on chromosome metaphase spreads with biotinylated probes and detection by Avidin-FITC followed by biotin-anti-Avidin and Avidin-FITC amplification was carried as described by Trask and Pinkel, Methods Cell Biol. 30:383-400 (1990), using a Zeiss Axiophot microscope. Chromosomes were counterstained with propidium iodide. The photomicrographs shown are representative of 95% of the metaphase spreads or interphase nuclei scanned in three independent experiments carried out with the human or the yeast probes. A single integration site was detected for the human sequences.

The same blots were also probed with the yeast Ty repetitive element sequence to detect the presence of yeast genomic DNA sequences in the ESY clones (Figure 12 D). Whereas some of the clones were found to contain most of the Ty-containing fragments present in the parental yeast strain, some of the clones were found to have a very small fraction, if at all, of the Ty-containing fragments. These results indicate that in some ES clones, although the YAC DNA is integrated intact, little or no yeast

chimeric mice. ESY cells were microinjected into C57BL/6J mouse blastocysts, and chimeric mice were generated as previously described. Chimeric males were mated with C57BL/6J females and germline transmission was determined by the presence of agouti offspring. Genomic DNA prepared from the tails of the chimeric mice were analyzed for the presence of the yHPRT DNA in the mouse genome by PCR analysis. The presence of the YAC left arm was analyzed using the two priming oligonucleotides, 5' TTCTCGGAGCACTGTC CGACC and 5' CTTGCGCCTTAAACCAACTTGGTACCG, which were derived, respectively, from the pBR322 sequences and the SUP4 gene within the YAC left vector arm. A 259 bp PCR product was obtained from the analysis of the yeast containing yHPRT and the ESY cell lines. PCR analysis of tail DNA prepared from 18 chimeric mice generated from ESY cell lines ESY3-1 ESY3-6 and ESY5-2, gave rise to the expected PCR product, thus indicating the presence of the YAC left vector arm in the genome of the chimeric mice.

6. Germline transmission of yHPRT

Chimeric males, with coat color chimerism of 30-60%, derived from the ESY cell lines ESY3-1 and ESY5-2 were set up for mating for germline transmission evaluation, i.e. to determine whether the genetic modification was passed via the germ cells (sperm or oocytes) to the progeny of the animals. Three of the chimeric ESY3-1 derived males, 394/95-1, 394/95-2 and 411-1 transmitted the ES cell genome to their offspring at a frequency of 20%, 30% and 30%, respectively. Southern blot analysis of tail DNA from the agouti pups indicated the presence of the yHPRT in the genome of three mice, 4-2, 4-3 and 5-1, derived from the 394/395-2 chimera. The Alu profile obtained from such analysis was indistinguishable from that of the parent ES3-1 cell line (Figure 14 C), demonstrating that the 680 kb human insert was transmitted faithfully through the mouse germline.

large molecular weight DNA fragment into ES cells and that such molecules are stably and functionally transmitted through the mouse germline. The Alu profiles, complemented by PFGE analysis and in situ hybridization for some of the ES clones, strongly argue that the majority of the clones contained virtually all the human insert in unrearranged form (i.e. in "germline configuration"), with a high frequency of clones (40%) also retaining both YAC arms. The significant uptake of yeast genomic DNA was not detrimental to proper differentiation of ES cells in vitro and in vivo and did not prevent germline transmission or gene expression. By these methods, one can transmit large fragments of genomic DNA as inserts into non-human animal genomes, where the inserts may be transmitted intact by germline transmission. Therefore, a wide variety of xenogeneic DNA can be introduced into non-human hosts such as mammals, particularly small laboratory animals, that may impart novel phenotypes or novel genotypes. For example, one can provide in small laboratory animals genes of a mammal, such as a human, to study the etiology of a disease, the response to human genes to a wide variety of agents. Alternatively, one can introduce large loci into a mammalian host to produce products of other species, for example humans, to provide human protein sequences of proteins such as immunoglobulins, T-cell receptors, major histocompatibility complex antigens, etc.

Introduction of heavy chain YAC A287-C10 and kappa chain YAC A80-C
into ES cells and Embryos

Yeast containing the human heavy chain YAC A287-C10 targeted with pLUTO (yA287-C10) were spheroplasted and fused with the HPRT-deficient ES cell line E14.1TG3B1 as described above. Ten HAT-resistant ES (ESY) clones (2B, 2C, 2D, 3A, 3B, 5C, 1125A, 1125E, 100/1500 and 100/4000) were picked and were expanded for DNA analysis.

Production of human Ig by chimeric mice by Introduction of Human Ig using Homologous Recombination

As an alternative approach to that set forth in Examples I-VI, human Ig genes are introduced into the mouse Ig locus by replacing mouse heavy and light chain immunoglobulin loci directly with fragments of the human heavy and light chain loci using homologous recombination. This is followed by the generation of chimeric transgenic animals in which the embryonic stem-cell derived cells contribute to the germ line.

A. Construction of human heavy chain replacement vector.

The replacing human sequences include the SpeI 100 kb fragment of genomic DNA which encompasses the human VH6-D-J-Cμ-Cδ heavy chain region isolated from a human-YAC library as described before. The flanking mouse heavy chain sequences, which drive the homologous recombination replacement event, contain a 10 kb BamHI fragment of the mouse Cε-Cα heavy chain and a 5′ J558 fragment comprising the 5′ half of the J558 fragment of the mouse heavy chain variable region, at the 3′ and 5′ ends of the human sequences, respectively (Figure 16). These mouse sequences are isolated from a mouse embryo genomic library using the probes described in Tucker et al. (1981), PNAS USA, 78: 7684-7688 and Blankenstein and Krawinkel (1987, supra), respectively. The 1150 bp XhoI to BamHI fragment, containing a neomycin-resistance gene driven by the Herpes simplex virus thymidine kinase gene (HSV-tk) promoter and a polyoma enhancer is isolated from pMC1Neo (Koller and Smithies, 1989, supra). A synthetic adaptor is added onto this fragment to convert the XhoI end into a BamHI end and the resulting fragment is joined to the BamHI mouse Cε-Cα in a plasmid.

From the YAC clone containing the human heavy chain locus, DNA sequences from each end of the insert are recovered either by inverse PCR (Silverman et al. (1989), PNAS, 86:7485-7489), or by plasmid rescue in E. coli,

Mice containing the human immunoglobulin locus are mated to mice with inactivated murine immunoglobulin genes to generate mice that produce only human antibodies. Starting with four heterozygous strains, three generations of breeding are required to create a mouse that is homozygous for inactive murine kappa and heavy chain immunoglobulins, and heterozygous for human heavy and kappa chain immunoglobulin loci. The breeding scheme is shown in Figure 17.

EXAMPLE IX

Production of Human Monoclonal Antibodies

A. Immunization of mice

Germline chimeric mice containing integrated human DNA from the immunoglobulin loci are immunized by injection of an antigen in adjuvant. The mice are boosted with antigen 14 days after the primary immunization, repeated after 35 and 56 days. A bleed is done on the immunized animals to test the titer of serum antibodies against the immunizing antigen. The mouse with the highest titer is sacrificed, and the spleen removed.

B. Fusion of splenocytes

Myeloma cells used as the fusion partner for the spleen cells are thawed 6 days prior to the fusion, and grown in tissue culture. One day before the fusion, the cells are split into fresh medium containing 10% fetal calf serum at a concentration of 5×10^5 cells/ml. On the morning of the fusion the cells are diluted with an equal volume of medium supplemented with 20% fetal calf serum and 2X OPI (3 mg/ml oxaloacetate, 0.1 mg/ml sodium pyruvate and 0.4 IU/ml insulin) solution.

After sacrificing the mouse, the spleen is aseptically removed, and placed in a dish with culture medium. The cells are teased apart until the spleen is torn into fine pieces and most cells have been removed.

permitted with a human host. The resulting B-cells may then be used for immortalization for the continuous production of the desired antibody. The immortalized cells may be used for isolation of the genes encoding the immunoglobulin or analog and be subjected to further molecular modification by methods such as in-vitro mutagenesis or other techniques to modify the properties of the antibodies. These modified genes may then be returned to the immortalized cells by transfection to provide for a continuous mammalian cellular source of the desired antibodies. The subject invention provides for a convenient source of human antibodies, where the human antibodies are produced in analogous manner to the production of antibodies in a human host. The animal host cells conveniently provide for the activation and rearrangement of human DNA in the host cells for production of human antibodies.

In accordance with the subject invention, human antibodies can be produced to human immunogens, eg. proteins, by immunization of the subject host mammal with human immunogens. The resulting antisera will be specific for the human immunogen and may be harvested from the serum of the host. The immunized host B cells may be used for immortalization, eg. myeloma cell fusion, transfection, etc. to provide immortal cells, eg. hybridomas, to produce monoclonal antibodies. The antibodies, antiserum and monoclonal antibodies will be glycosylated in accordance with the species of the cell producing the antibodies. Rare variable regions of the Ig locus may be recruited in producing the antibodies, so that antibodies having rare variable regions may be obtained.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

CLAIMS

WHAT IS CLAIMED IS:

1. An improved method for modifying the genome of a embryonic stem cell so as to contain, in the genome thereof, a xenogeneic DNA segment of at least 100 kb which method comprises:

combining under fusing conditions embryonic stem cells and yeast spheroplasts, said spheroplasts containing a yeast artificial chromosome (YAC) comprising said xenogeneic DNA segment and including a marker for selection, whereby said xenogeneic DNA segment becomes integrated into the genome of said embryonic stem cells; and

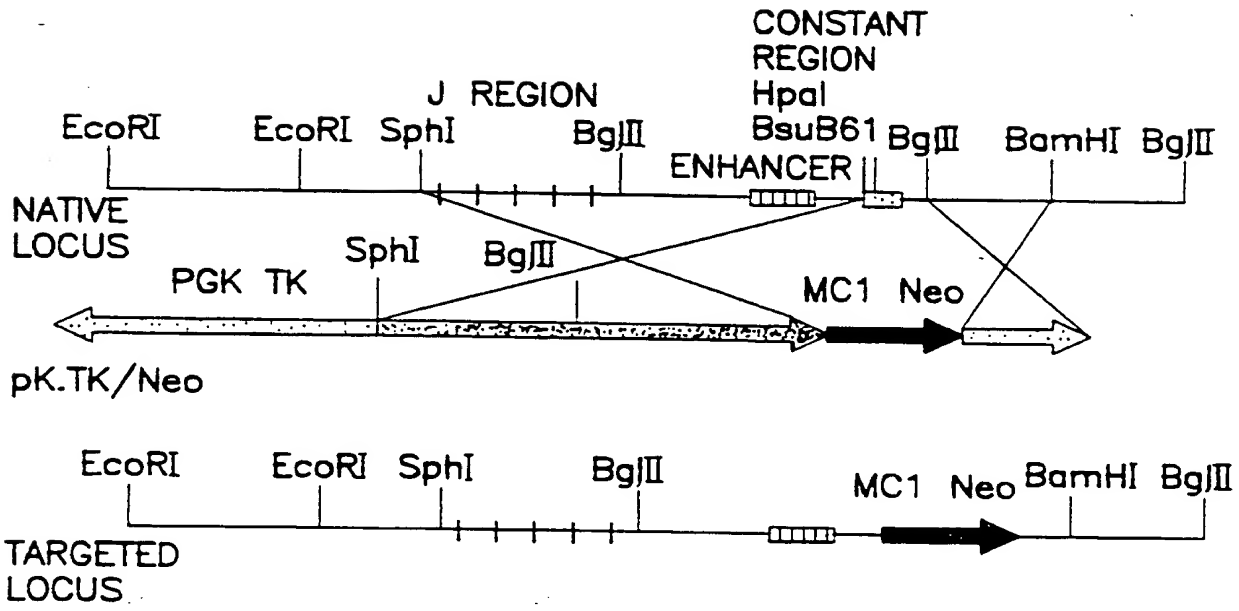
selecting for an embryonic stem cell carrying said xenogeneic DNA segment by means of the marker.

2. An improved method for producing a modified non-human animal, said animal having a xenogeneic DNA segment of at least 100 kb stably integrated into the genome of at least some cells of said animal, said method comprising:

combining under fusing conditions embryonic stem cells of said animal and yeast spheroplasts, said spheroplasts containing a yeast artificial chromosome (YAC) comprising said xenogeneic DNA segment and including a marker for selection, whereby said xenogeneic DNA segment becomes integrated into the genome of said embryonic stem cells;

selecting for embryonic stem cells carrying said xenogeneic DNA segment by means of the marker; and

transferring said selected embryonic cells into a host blastocyst, implanting said blastocyst in a pseudopregnant animal recipient, and allowing said blastocyst to develop to term to produce a chimeric animal carrying said xenogeneic DNA segment integrated into the genome of at least some cells of said animal.


CONSTANT
REGION
HpaI
BsuB61
J REGION
EcoRI
EcoRI
SphI
BglII
ENHANCER
BglII
BamHI
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NATIVE
LOCUS
SphI
BglII
PGK
TK
MC1
Neo
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Neo
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BglII
TARGETED
LOCUS

FIG. 5

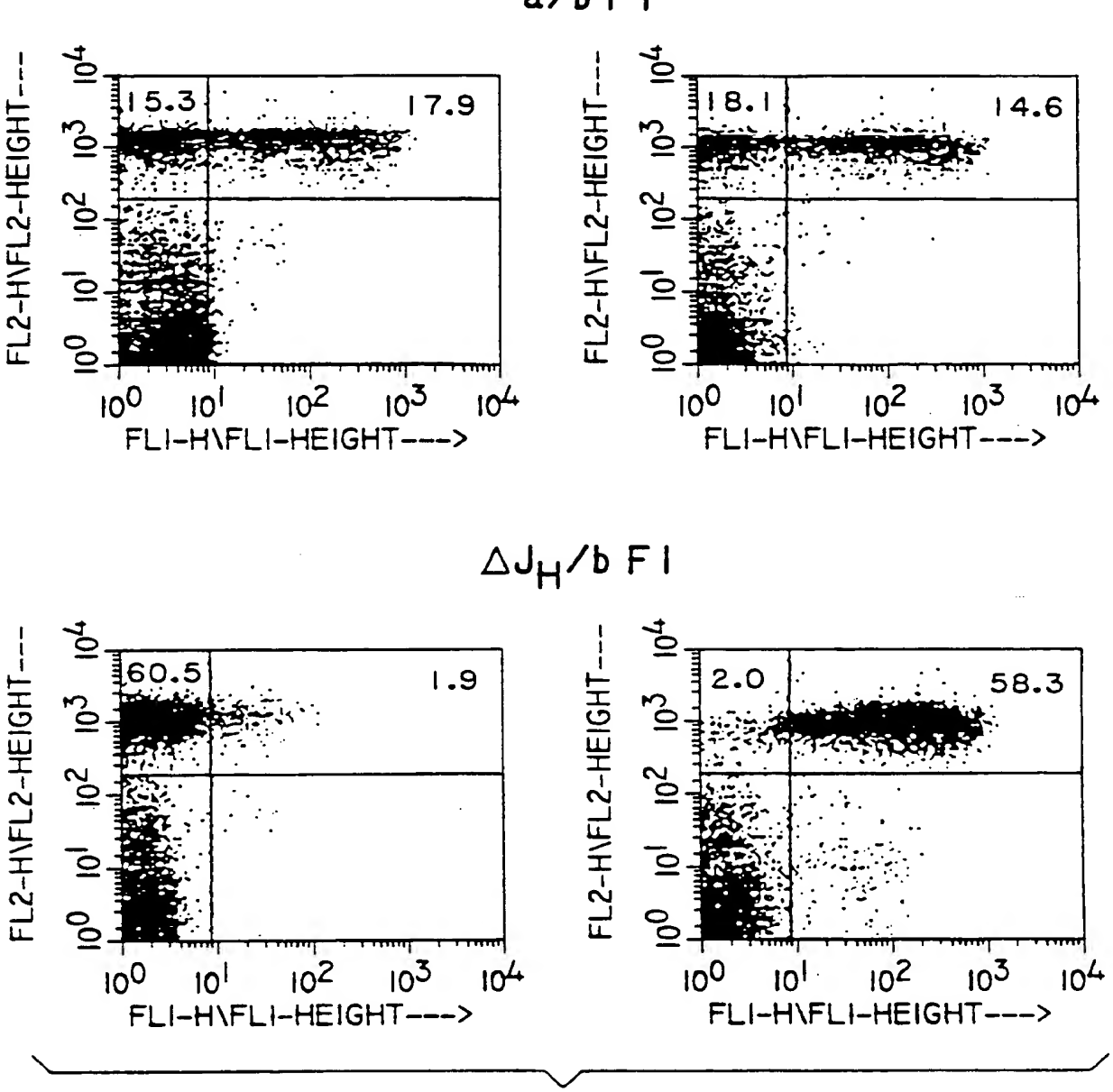
a/b F1
FL2-H\FL2-HEIGHT---
FLI-H\FLI-HEIGHT--->
15.3
17.9
18.1
14.6
ΔJH/b F1
60.5
1.9
2.0
58.3

FIG. 3-2

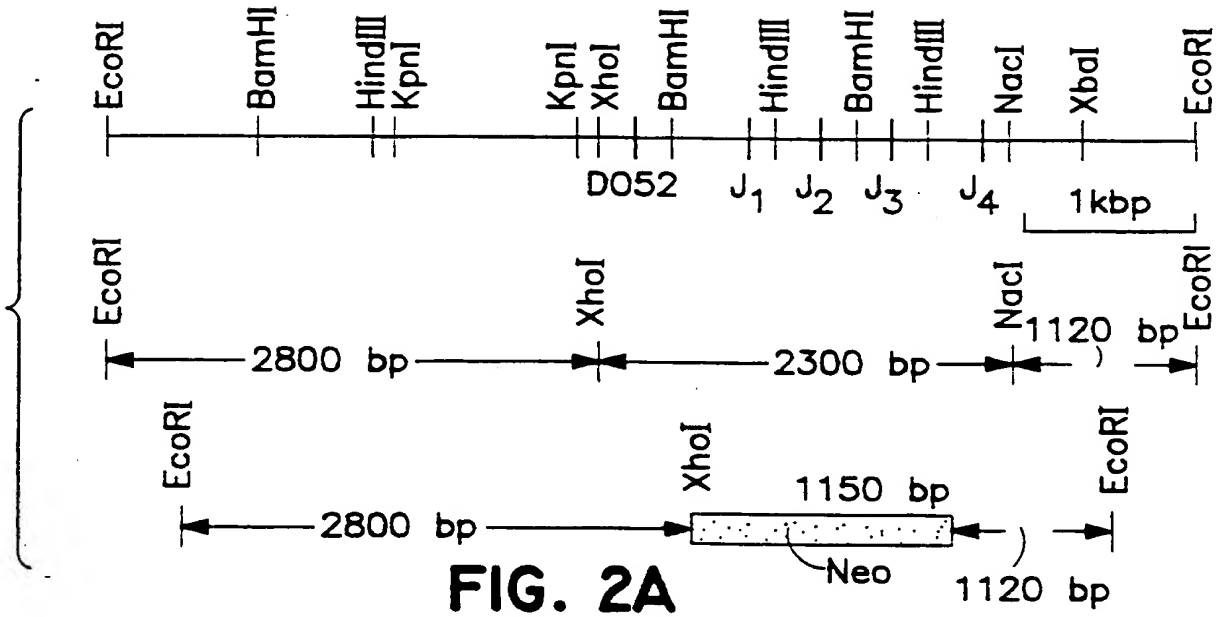

FIG. 2A

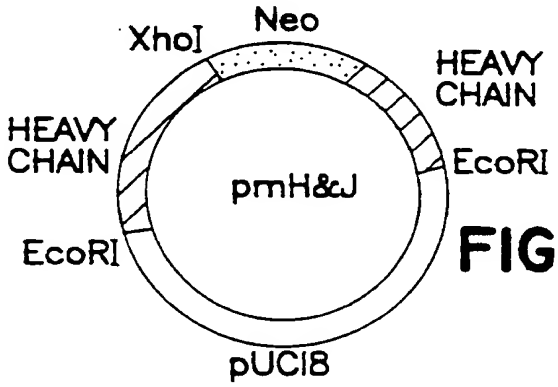

FIG. 2B

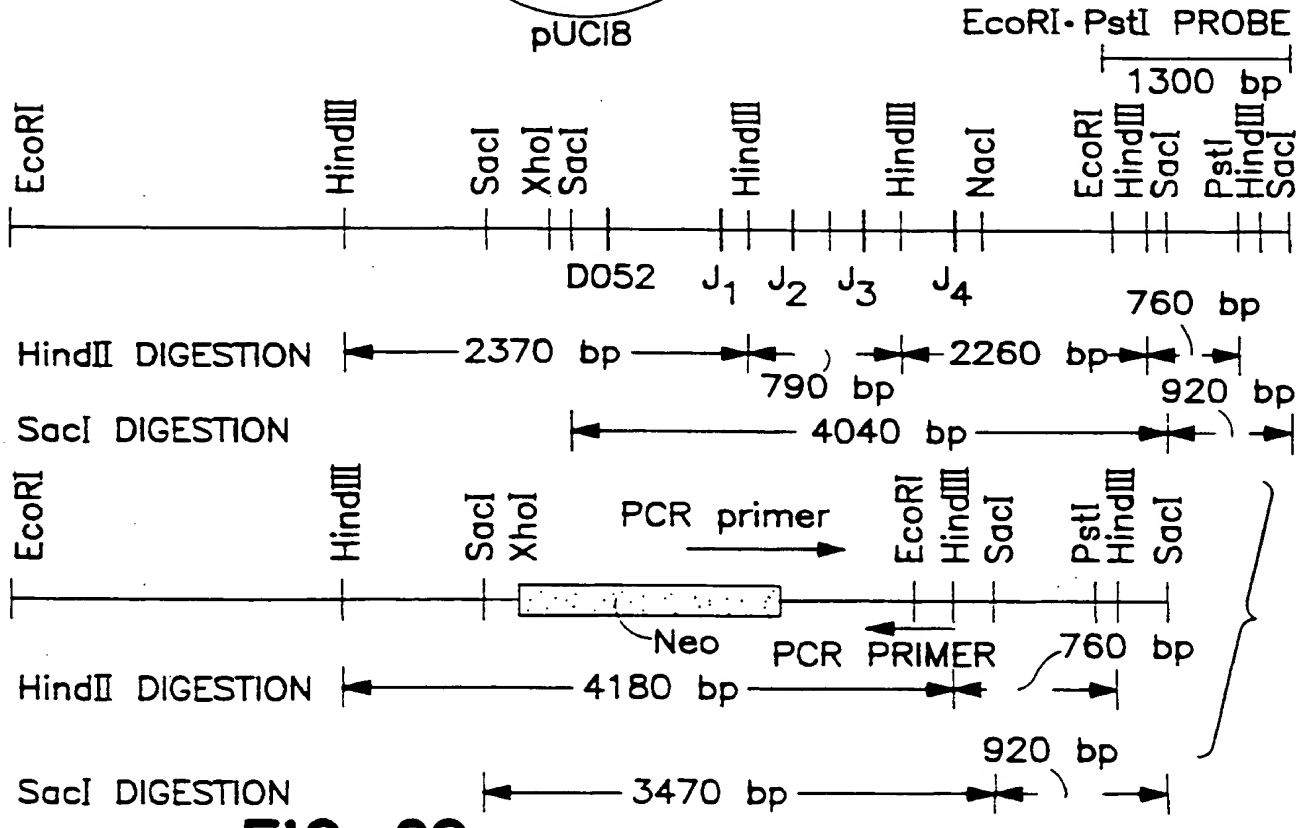

FIG. 2C

8. The stem cells of claim 6 which are of a rodent.

9. The stem cells of claim 8 which are murine.

10. The animal of claim 7 which is a rodent.

11. The animal of claim 10 which is a mouse.

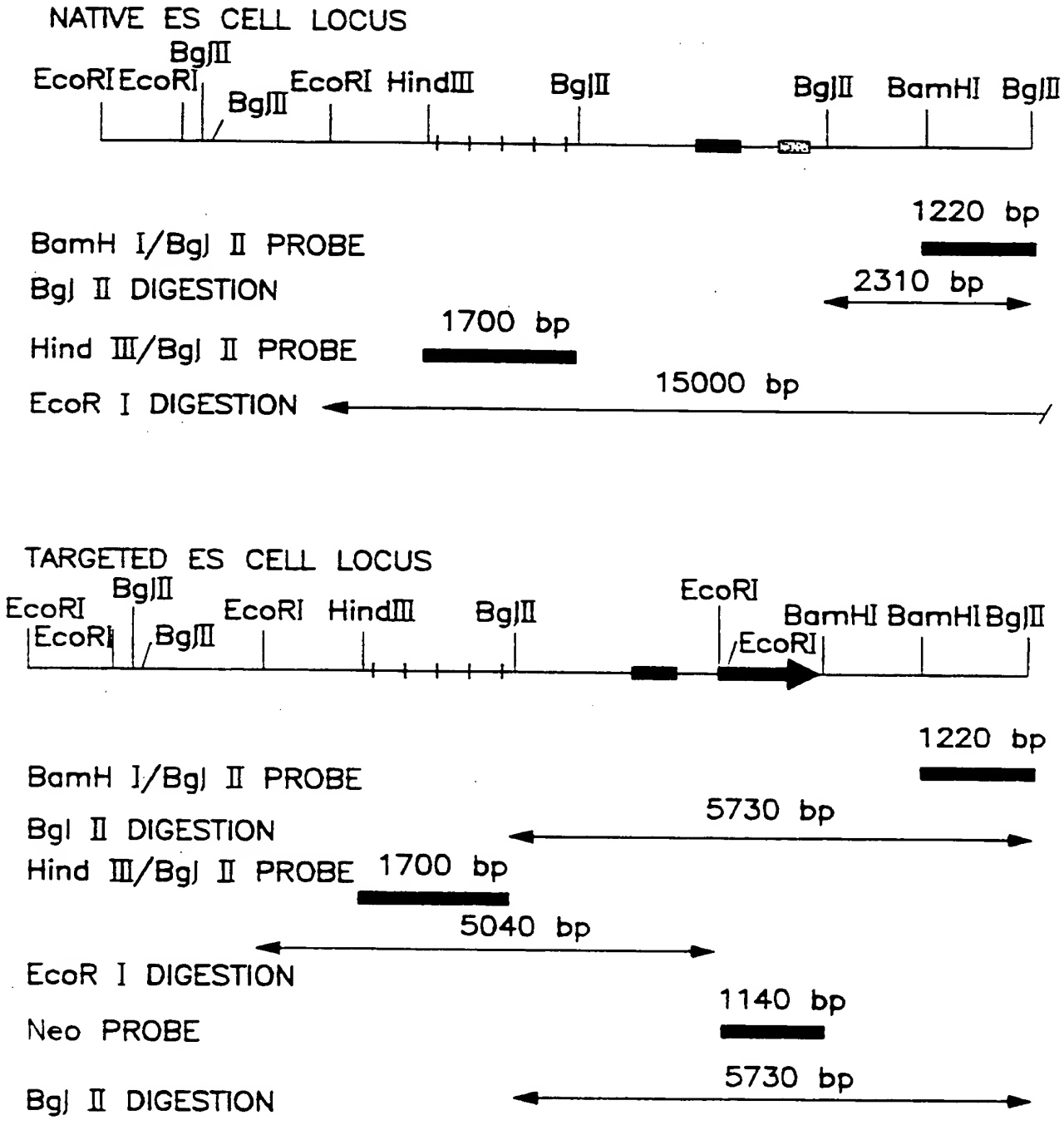
NATIVE ES CELL LOCUS
BglII
EcoRI EcoRI
BglII
EcoRI HindIII
BglII
BglII BamHI BglII
1220 bp
BamH I/Bgl II PROBE
Bgl II DIGESTION
2310 bp
1700 bp
Hind III/Bgl II PROBE
15000 bp
EcoR I DIGESTION
TARGETED ES CELL LOCUS
BglII
EcoRI
EcoRI
EcoRI HindIII
BglII
EcoRI
BglII
BamHI BamHI BglII
EcoRI
1220 bp
BamH I/Bgl II PROBE
5730 bp
Bgl II DIGESTION
Hind III/Bgl II PROBE
1700 bp
5040 bp
EcoR I DIGESTION
1140 bp
Neo PROBE
5730 bp
Bgl II DIGESTION

FIG. 7